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14. ABSTRACT <p>This project was to develop novel therapies for Parkinsons Disease (PD), to test the effects of human dopaminergic stem cells, and to utilize metabolic profiling to develop biomarkers for PD. We developed a new transgenic mouse model of PD by expressing the LRRK2 mutation, R1441G, in the full-length human LRRK2 protein utilizing a bacterial artificial chromosome (BAC). These mice develop a profound parkinsonian phenotype in which they become markedly slowed with a flexed postured and impaired functioning using the rotarod. They have impaired release of dopamine as assessed using microdialysis and despite a normal complement of dopaminergic neurons, these mice showed axonal pathology in which there was phosphorylated tau, which formed spheroids. We carried out studies of PINK1 in cultured neurons and demonstrated that PINK 1 deficiency results in mitochondrial impairment. We also made a knockout PINK1 mouse. We utilized HPLC coupled to coularray electrochemical detection to perform metabolomic profiling. We showed that we could separate the patients with LRRK2 mutations from idiopathic PD and controls. Similarly, we could separate gene positive at risk from both controls and LRRK2 mutation negative subjects. Lastly, we found that administration of triterpenoids which activate the Nrf2/ARE pathway are neuroprotective against MPTP. These studies have continued over the last year and we have been able to make further progress in this area in meeting the research goals outlined in our tasks.</p>					
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4. INTRODUCTION

This is a project, which has been ongoing since September of 2004. The grant initially was designed to study neuroprotective agents in an MPTP model of Parkinson's Disease (PD), as well as the pathophysiology of mitochondrial dysfunction in PD. We modified the proposal to characterize a new animal model of PD made by knocking out PINK1, a nuclear encoded kinase localized to mitochondria, and to study the effects of human dopaminergic stem cells in a 6-hydroxydopamine (6-OHDA) model of PD. More recently, we revised our efforts to focus on the development of metabolomic profiling to identify biomarkers for PD. These studies are ongoing and we have been able to make considerable progress in this area over the last year.

Outline of Research Goals:

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations can cause autosomal recessive PD.

Task 3: To utilize metabolomic profiling to develop biomarkers for PD.

Task 4: To determine the efficacy of human dopaminergic stem cells in the 6-hydroxydopamine model of PD.

5. BODY

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity. These studies have been largely completed although we extended them further in the past year. In the past year, we utilized a novel therapeutic approach using triterpenoids, which are agents which target the Nrf2/ARE pathway. We demonstrated that these compounds can induce antioxidant enzymes in normal fibroblasts, however, the ability to induce these enzymes glutathione-c transferase, NADPH quinone oxidoreductase and heme-oxygenase was blocked in fibroblasts knocked out for Nrf2/ARE. We studied the administration of a methylamide derivatized triterpenoid, designated TP224. We found that this compound produced excellent levels in the brain tissue following oral administration, and that these levels were within the range which produces therapeutic effects *in vitro*. They were able to produce levels as high as 90 nanomolar whereas one nanomolar concentrations are effective *in vitro* in inhibiting iNOS. Following administration of this compound to mice for one week, we administered MPTP on an acute dosing regimen in which the compound was given every two hours for 4 doses. This produced a 50-60% depletion of dopamine and its metabolites as well as marked reduction in dopaminergic neurons assessed using tyrosine hydroxylase immunocytochemistry. We showed that administration of TP224 was highly effective in significantly reducing the depletion of dopamine and its metabolites as well as the loss of tyrosine hydroxylase neurons. We also examined the compound in a chronic dosing regimen of MPTP toxicity in which it is administered over one month using an Alzet pump. We administered MPTP at a dose of 30mg/kg/per day. This produced a 50% depletion of dopamine within the striatum as well as a significant reduction in TH immunoreactive neurons. We were able to show that we could significantly protect against the loss of both dopamine in the striatum as well as dopaminergic neurons within the substantia nigra. Furthermore, we demonstrated that there was a production of alpha-synuclein aggregates within the cytoplasm of the dopaminergic neurons treated chronically with MPTP, following administration of the triterpenoid, the development of these aggregates was completely blocked. We also showed that we could attenuate increases in malondialdehyde, 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine as assessed using immunohistochemistry. These results were published in *Neurobiology of Disease*.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations can cause autosomal recessive PD and by overexpressing LRRK2 R1441G mutations in LRRK2 using BAC transgenesis.

We generated the PINK1 knockout mice. Correctly targeted ES cells were used to inject and generate PINK1 knockout mouse founders. We demonstrated that there were mild defects in motor behavior. We also found impaired dopamine release using microdialysis. We observed that there are reduced numbers of phosphorylated proteins on 2-dimensional gel electrophoresis. One of the proteins is the protein Omi which has

been shown to be phosphorylated by PINK1. We confirmed this result and we are continuing to identify other substrates for PINK1.

We also utilized BAC transgenesis to develop a novel model of PD based on mutations in LRRK2. The BAC clone was correctly isolated and then underwent site-directed mutagenesis. We verified the construct using sequencing. The construct was then injected into founder mice and we were able to identify viable offspring. These mice developed normally. They however, by 10 months of age developed a profound parkinsonian phenotype. We could first detect abnormalities of movement as early as 6 months of age. By 10 months of age, the animals were largely immobile and showed a marked flex posture with slowed movements. They were impaired on rearing and open field activity. They were also impaired on the pole test and rotarod testing. We used microdialysis to demonstrate that at baseline the production of dopamine after administration of nomphensine was impaired in the LRRK2 R1441G mice. We made counts of dopaminergic neurons, which showed that they were unaltered. The neurons however, did show some very mild shrinkage. In addition, there was a loss of dopaminergic axons in the substantia nigra reticulata. We found that the dopaminergic axons in the striatum showed marked abnormalities with axonal spheroids. Using immunostaining, they showed phosphorylated tau as detected with the AT8 antibody. This result is consistent with the observations made by Matt Farrer and colleagues at the Mayo Clinic Jacksonville. We are continuing to characterize these mice and we have commenced doing therapeutic studies to determine whether they will prove to be a useful model for developing new treatments for PD.

Task 3: To utilize metabolomic profiling to develop biomarkers for PD. The primary aim of our ongoing studies as well as the existing protocols to determine whether neurochemical markers in blood or spinal fluid can be used to make an early diagnosis or to follow the progression of PD. We have previously demonstrated that we are able to completely separate unmedicated PD subjects from patients with dopaminergic medications as well as normal controls. Our initial studies were carried out on 25 controls and 66 PD subjects. We were able to detect as many as 2000 peaks using HPLC with electrochemical coulometric array detection. These initial studies were published in Brain. We have subsequently carried out further studies of patients and gene carriers with the LRRK2 mutations, which is responsible for autosomal dominant inherited PD although with partial penetrance which is age-dependent. We were able to identify a number of variables which differentiated LRRK2 PD subjects from idiopathic PD subjects. We were also able to separate LRRK2 PD subjects from family members who were carriers of the genetic defect as well as patients who are controls. Our results show that we can clearly separate PD patients with LRRK mutations from idiopathic PD and controls. We also carried out studies of predictive testing and showed that we were able to separate gene positive patients from LRRK2 carriers as well as LRRK2 negative patients within the same families. We are now working on structural elucidation of the remaining analytes. We have developed a technique to utilize mass spectroscopy in combination with coulometric array electrochemical detection. This involves injecting a sample, which goes through a reverse phase column and is then split post column to be analyzed both by the electrochemical and mass spectrometric detectors in parallel. We

are also concentrating peaks so that we can subject them to mass spectroscopy again to attempt to identify the compounds, which are responsible for the separation of our treatment groups. We are also expanding our studies to look at other parkinsonian disorders which mimic idiopathic PD. We are carrying out other studies to determine whether we can see any other markers within the electrochemical profiles which correlate with other well established markers of oxidative damage such as F2 isoprostanes and 8-hydroxy-2-deoxyguanosine. We have identified abnormalities in the xanthine uric acid pathway. The PD patients have lowered uric acid as previously described.

Task 4: To determine efficacy of human dopaminergic stem cells in the 6-hydroxy dopamine model of PD. These studies were completed. We were able to show that the stem cells survived. They were utilized to develop highly purified human embryonic stem cell derived dopaminergic neurons using dopaminergic neurogenesis from human ES cells. We utilized a new technique which involved co-culture with telomerase immortalized human mesencephalic astrocytes during induction of a dopaminergic phenotype using sonic hedgehog and FGF8. Utilizing these enriched dopaminergic neurons, we were able to achieve a substantial long lasting restitution of motor function in 6-OH dopamine lesioned adult rats. We also showed that there was affective generation of TH-positive neurons in all six animals studied. We examined BDRU incorporation which showed that there was a small percentage of neurons which were still dividing consistent with evidence of mitosis. These are important studies which have been published in *Nature Medicine*.

We have recently worked on developing induced pluripotent stem cells from fibroblasts from PD subjects. These studies are ongoing and are being done in collaboration with Dr. Lorenz Studer at Memorial Sloan Kettering Institute. He has been able to successfully produce IPS dopaminergic neurons from patients with both idiopathic PD as well as controls. We have also given him samples from patients with known LRRK2 mutations such as the G2385R mutation. A number of these fibroblasts have been provided by Dr. Jan Aasley from Norway and we have demonstrated that it is possible to ship these fibroblasts to New York to Norway and still derive viable IPS induced dopaminergic neurons.

6. RESEARCH ACCOMPLISHMENTS

- A. We demonstrated that several novel pharmacologic agents exert neuroprotective effects in the MPTP model of PD. In particular, in the most recent grant, we have demonstrated that triterpenoids, which activate the Nrf2/ARE pathway are highly effective in both acute and chronic MPTP models of PD. This suggests that these agents are worthwhile for further development for treatment of PD patients.
- B. We have developed a knockout model of PINK1, which we are continuing to study. We have also developed a BAC R1441G LRRK2 transgenic mouse model for PD. These animals are undergoing further characterization in order to carry out studies of therapeutic interventions. We have initiated studies of a number of promising therapeutic compounds, which are continuing to progress.
- C. We showed that HES-derived dopaminergic neurons exert beneficial effects in the 6-hydroxydopamine models of PD.
- D. We have generated IPS cells and dopaminergic neurons starting with fibroblasts from patients with idiopathic PD as well as patients with defined mutations in LRRK2. We are now studying these fibroblasts induced dopaminergic neurons further to determine their phenotype with regard to dopamine, mitochondrial function and production of reactive oxygen species.
- E. We have continued metabolomic profiling and now have shown that we can separate unmedicated PD patients from controls as well as medicated PD patients from controls. This work has been published. We have also showed that a number of specific metabolites are altered including 8-hydroxy-2-deoxyguanosine and reduced uric acid. We have also found abnormalities in both hypoxanthine and xanthine. We can separate idiopathic PD patients from patients with the LRRK2 mutation and in addition, we can separate LRRK2 gene positive carriers from controls and LRRK2 mutation negative patients. This provides evidence that it may be possible to screen patients early in life and determine whether they will eventually be at high risk of developing PD.

7. REPORTABLE OUTCOMES

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8. CONCLUSION

We have accomplished much of our original research goals. We have characterized a number of agents which show protection against MPTP toxicity, and in particular we have recently shown that Nrf2/ARE pathways agonists are highly effective. We developed new transgenic mouse models of PD by knocking out PINK1 and by overexpression of R1441G LRRK2 induced PD in mice. These mice show a very robust parkinsonian phenotype which is responsive to dopaminergic agents. We are developing these for study of therapeutic agents. We have continued our metabolomic profiling studies of PD patients and we have found that we can identify unique biomarkers in patients with LRRK2 mutations. We completed studies of transplantation of human ES cell derived dopaminergic neurons into 6-hydroxydopamine lesioned rat model of PD. We have shown successful restitution of behavioral abnormalities. We have also developed induced pluripotent stem cells from human fibroblasts with known parkinsonian mutations as well as idiopathic PD.

9. REFERENCES

None.

10. APPENDICES

None



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October 8th, 2010

Juanita Livingston
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Re: Annual Report W81XWH-04-1-0802

Dear Ms. Livingston:

Attached please find our annual report covering research for the period September 01, 2009 to August 31st, 2010.

Sincerely yours,


M. Flint Beal, M.D.